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# INDUCTION OF BLAST DISEASE RESISTANCE UPON SEED PRIMING WITH *PSEUDOMONAS* SP. IN FINGER MILLET

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## Abstract

An efficient mechanism to induce disease resistance in finger millet plants against the dreadful blast disease caused by *Magnaporthe grisea* has been established in the present study. The study involved the characterisation of the plant growth promoting bacteria (PGPR) fluorescent *Pseudomonas* JUPC113 and JUPW121 isolated from cotton and wheat rhizospheres respectively. The performance of the isolates was better with respect to growth promotion and in vitro fungal inhibition against the pathogen. JUPW121 showed highest growth promotion (vigour index of 3064.00 ± 3.06) and fungal inhibition of 75 mm after ten days of incubation. The greenhouse studies revealed that seed priming with JUPC113 and JUPW121 enhanced the rate of disease protection compared to that of mock-control plants. The phenolic acid content increased in primed plants decreased in the mock-control plants in comparison with water control plants whereas the chlorophyll content remained same in primed plants but decreased in mock-control plants. n primed, whereas was. When the leaf tissues were stained with a mixture of acridine orange and ethidium bromide and observed under fluorescent microscope, the dead and necrotic cells (mock-control) appeared orange-red whereas the living (primed) cells appeared green. Scanning electron micrographs evidenced pathogen infestation by appearance of appressorium and conidial structures in the lesions in finger millet leaves. Thus, the present study focuses on induction of disease resistance in finger millet plants via seed priming with PGPR which is the present day focus due to its beneficial, sustainable and eco-friendly approach.

***Keywords*:** Chlorophyll, Phenolic acids, priming, resistance, SEM

## Introduction

Food security is of great concern, due to increased population, scarce rainfall and inadequate cultivable land area. Recent report by United Nations (FAO, 2018) indicates that malnourishment is a threat to the developing countries. The need for increased food production to meet the increasing world population can be achieved by switching to the less water-requiring, stress tolerant crops, which can be developed by conventional or breeding approaches. Finger millet [*Eleusine coracona* (L). Gaertner] is a major millet crop with beneficial nutritional properties. High calcium content (344 mg.100 $g^{-1}$) in finger millet is one of the main concerns of using the products of these edible seeds, in countries of Africa and South Asia. In India, Karnataka is the largest producer of finger millet with 58% of global production (Chandra *et al*., 2016). Finger millet is known to be infected by 20 different pathogens, amongst which blast disease caused by *Magnaporthe grisea* (T.T. Hebert) M.E. Barr., is known to be most dreadful accounting for ~80% of the total yield loss. This pathogen is ranked number one in the recent years with respect to the plant fungal pathogens (Dean *et al*., 2010). There are various strategies employed for combating the dreadful disease. Use of beneficial microbes, plant growth promoting rhizobacteria (PGPR) involving increased plant growth promoting traits is of interest in the present day scenario. These microbe-mediated disease inducing mechanisms are eco-friendly, sustainable, reproducible and cost effective approaches (Radjacommare *et al*., 2004; Patil *et al*., 2016; Kumudini *et al*., 2017; Jayamohan *et al*., 2018; Sekar *et al*., 2018).

Previous studies (Radjacommare *et al.,* 2004; Kumar and Kumar 2011; Patil *et al.,* 2016; Sekar *et al.,* 2018) have been carried out on induction of disease resistance

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in finger millet plants using *Pseudomonas* species, which revealed the increase in resistance in primed plants. In the present study the objectives were (i) to characterise the bacterial isolates on *in vitro* growth promotion and antifungal activity, (ii) to induce blast disease resistance by priming the susceptible finger millet seeds with the bacterial isolates and (iii) to determine the different defense molecules attributing towards induction of disease resistance in finger millets.

## Materials and Methods

In the present study, susceptible finger millet (Indaf 9, *var*.) seeds were obtained from MAS Laboratory, University of Agricultural Studies, Bengaluru, Karnataka, India. The blast pathogen *M. grisea* (MTCC-1477) was procured from CSIR-IMTECH, Chandigarh, India and maintained on oat meal agar until use. Fluorescent *Pseudomonas* JUPC113 and JUPW121 isolated from cotton and wheat rhizospheres respectively were further characterized for its potential plant growth promotion and *in vitro* antifungal activity.

***In vitro* Growth Promotion and Antifungal Activity :** The isolates JUPC113 and JUPW121 were grown in Luria Bertani (LB) broth for 48 h at 37 ± 2°C under shaking conditions (180 rpm). For *in vitro* growth promotion studies, the 48 h old cultures were centrifuged at 6,000 × g for 10 min at 4 ± 2°C and resuspended in 0.85% NaCl with a concentration of $4 \times 10^8$ CFU.ml$^{-1}$ and used for priming seeds. Finger millet seeds were rinsed with running tap water followed by surface sterilization using 0.2% sodium hypochlorite and again rinsed with distilled water. The seeds were then treated with bacterial cell suspension for 10 h at room temperature (27 ± 2°C) with intermittent shaking. The cell suspension was decanted, seeds blot dried and used for germination studies by top of the paper methodq (ISTA, 1993). Distilled water seeds were maintained as control. After ten days, root and shoot length were measured and germination percentage and vigour index (VI) were calculated (Babu *et al.,* 2015). To study the antifungal activity by well diffusion method (Zhongzheng *et al*., 2009), the cultures were inoculated into the wells bored in the potato dextrose agar (PDA) plates inoculated with the *M. grisea* spores. The zone of clearance was observed after 7 d of incubation at 27 ± 2ºC.

**Molecular Identification :** Species-level identification of the isolates JUPC113 and JUPW121 was carried out by partial sequencing of the 16s rRNA (Chromous Biotech Pvt. Ltd., Bengaluru). Raw data in the Fasta format was fed into the software nucleotide Basic Local Alignment Search Tool (nBLAST) from National Centre for Biotechnology Information (NCBI), USA and the best similarity with more sequence overlapping was chosen. The sequences were then submitted to the NCBI library.

**Induction of Disease Resistance :** Primed finger millet seeds were transferred to earthen pots containing sand and soil in the ratio 1:1 and sterilized at 15 lb pressure for 30 min before use. The seeds treated with distilled water were used as control throughout the study. The treatments were maintained in randomised block design (RBD) under greenhouse conditions in triplicates (temp. 28/20 ± 2°C and 65 ± 2% RH) until two-leaf stage. The plants were challenge inoculated with *M. grisea* spore ($1 \times 10^6$ spores.mL$^{-1}$) suspension. A set of control plants were also challenge inoculated and was maintained as Mock-control. Another set of plants were un-challenged and maintained as Water-control. After 20 days post inoculation (dpi), the plants were assessed for the lesions on the leaves for disease incidence.

**Seed-Priming Induced Defense Mechanism :** The different attributes towards host resistance against the blast disease was assessed by sampling leaves (treated and control) after 72 hours post inoculation (hpi). Powdered leaf tissue (0.5 g) was used to extract the total phenolic content using 3.0 mL of 80% methanol (Arnon, 1949). Five millilitre of sodium carbonate (2%) was added to 1.5 mL of the methanolic leaf extract and incubated at room temperature (27 ± 2ºC) for 5 min. and incubated with 0.2 mL of Folin-Ciocalteu reagent for 10 min under dark conditions. The total phenolic content was determined by reading the absorbance at $_{1760nm}$ and expressed as µg.g$^{-1}$ FW against the catechol standard (Zieslin and Ben-Zaken, 1993). 0.1 g of each of the leaf samples (primed and control) were ground using liquid nitrogen in a frozen mortar and pestle to extract total chlorophyll with 5.0 mL of 80% acetone (McKinney, 1938). The mixture was filtered through a muslin cloth and total volume made upto 10.0 mL using 80% acetone. Chlorophyll a and b was determined by reading the absorbance at $\lambda_{665}$ and $\lambda_{650}$ nm respectively. The total chlorophyll content was determined by the formula.

Total chlorophyll content = $\lambda_{665}$ (6.45) + $\lambda_{650}$ (17.72)

**Cell Death and Scanning Electron Microscopic Studies :** The effect of pathogen infestation on the mock and primed plants were detected by dual stains, acridine orange and ethidium bromide as described by Ciniglia *et al*., (2010) with minor modifications. The leaves were collected after 72 hpi and treated with a dye mixture of acridine orange (100 ¼g.ml$^{-1}$) and ethidium bromide (100 ¼g.ml$^{-1}$) prepared in phosphate buffer saline (pH 8.0) and mixed gently. The leaves were then mounted with

glycerol and observed under the fluorescence microscope (with the UV filter block of $\lambda_{350\text{-}410nm}$) for differentiating the dead (orange) and live (green) cells. The pathogen progression was observed by capturing the scanning electron micrographs from Hitachi Table top (TM3030 plus) with Variable Pressure (VPSEM) Detectors. The leaf samples were imaged without any dehydration and the pathogen ingression was observed without any chemical coating and magnification ranged from 400-500x.

**Statistical Analysis :** The results of biochemical studies are represented as mean ± standard error and subjected to analysis of variance (ANOVA). The data are compared using Duncan's non parametric similarity co-efficient at $p \leq 0.05$ using IBM SPSS statistics 20 package. Graph Pad Prism Version 8.1.0 was used for statistical analysis as well graph generation.

## Results and Discussion

Blast disease caused by *M. grisea* can be controlled by use of certain beneficial bacteria in rice, wheat and finger millet as reported by Radjacommare *et al*., (2004), Kumar and Kumar (2011) and Sekar *et al*., (2018). The study involved the characterisation of isolates for growth promotion by top of the paper method and antifungal activity against *M. grisea* by well diffusion method. JUPW121-primed plants showed increased root and shoot length compared to the JUPC113-primed plants. These were highly significant with the water-treated (control) plants (Fig. 1.), indicating the role of seed priming by the bacterial inducers. After seven days of inoculation, the ability of the isolates to inhibit the fungal growth under *in vitro* conditions was noted. The increased zone of clearance was exhibited by JUPW121 (75 mm) compared to that of the JUPC113 (58 mm). Control (0 mm) was uninoculated LB broth as indicated in (Fig. 2.), Hence, results of the preliminary characterisation indicate that these bacteria possess potential plant growth promoting and fungal inhibition traits, which are a prerequisite for biocontrol studies. These bacteria were further used in greenhouse studies to induce disease resistance. The partial 16s rRNA sequencing resulted in the identity with *Pseudomonas aeruginosa* species for both JUPC113 and JUPW121 with a similarity of 99%. The accession numbers of the isolates were KX00601 and KX00602 for JUPC113 and JUPW121 respectively.

The greenhouse studies revealed that these isolates influenced the disease protection in primed plants compared to the un-primed control plants. Symptomatology (Fig. 3.), indicated that the priming enhanced disease protection which was nullified in the


**Fig. 1 :** Effect of fluorescent *Pseudomonas* – treatment on root and shoot length of finger millet seeds after 10 days.

Arrows indicate the length of the roots corresponding to the centimeter scale.

mock-control plants. The water-control plants remained normal without any symptoms as the plants were not exposed to challenge inoculation. The lesions were more prevalent in the mock-control plants, where as JUPW21-primed leaves showed lesser diseased lesions followed by the JUPC113-primed plants. This was in accordance with the disease score and protection rate (Table 1.). Thus from these *in vitro* and greenhouse studies, it is

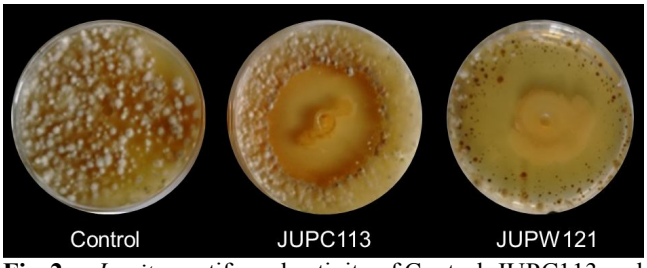


**Fig. 2 :** *In vitro* antifungal activity of Control, JUPC113 and JUPW121 against the blast pathogen *M. grisea* after 7d of inoculation.

**Table 1:** Efficiency of shortlisted fluorescent *Pseudomonas* isolates on challenge inoculation of primed ragi plants for induction of resistance against leaf blast disease under greenhouse conditions

| Treatments | Disease Incidence | Disease Protection (%) |
|---|---|---|
| JUPC113 | $0.27\pm0.02^{b}$ | $89.59\pm0.29^{c}$ |
| JUPW121 | $0.21\pm0.04^{d}$ | $92.03\pm0.04^{a}$ |
| Mock | $0.24\pm0.03^{c}$ | $91.03\pm0.04^{b}$ |
| Water | $2.67\pm0.33^{a}$ | $0.00\pm0.00^{d}$ |

*Values presented are mean ± standard error (n=3). Means were compared between treatments by DMRT. Different letters indicate significant differences among treatments ($pd \leq 0.05$).

more evident that JUPW121-priming has enforced the resistance mechanism after pathogen challenge compared to that of the JUPC113-primed plants. The *in vitro* results also indicate that JUPW121 is at a higher end compared to JUPC113 in growth promotion and fungal inhibition.

Studies (Jain *et al*., 2015; Patil *et al*., 2016; Kumudini *et al*., 2017; Jayamohan *et al*., 2018; Sekar *et al.,* 2018) have reported that the host cells enforce various chemical signals which mount resistance in the host after pathogen ingression. Reactive oxygen species, phenolic compounds, hormones and other cell wall bound molecules are such chemicals which are enhanced during pathogen invasion (Shalaby and Horwitz, 2014; Patil *et al*., 2016; Kumudini *et al*., 2017; Jayamohan *et al*., 2018). One of such

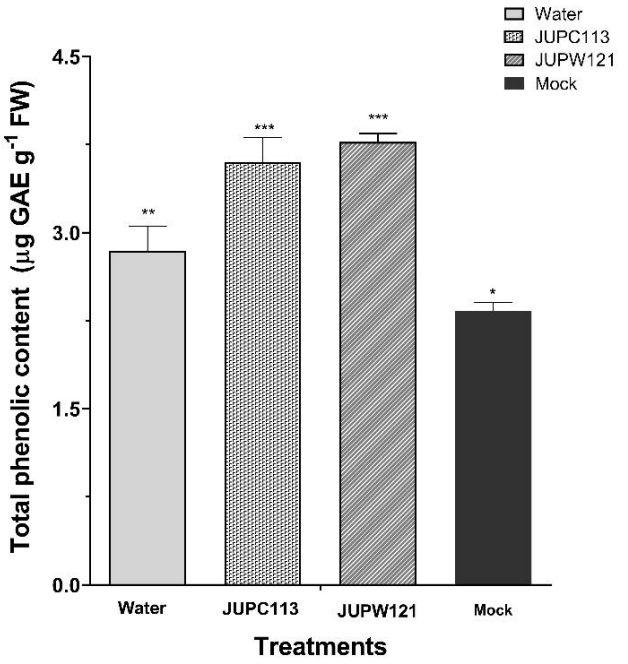


**Fig. 4:** Total phenolic content in the methanolic extracts of leaves collected from different treatments after 72 hpi in finger millet plants. Values presented are mean ± SE (n=3), the values were compared and significant difference ($p \leq 0.05$) among treatments are indicated with stars.

**Table 2:** Total chlorophyll content expressed in $\mu g.gFW^{-1}$ in finger millet plants

| Treatments | Chlorophyll content |
|---|---|
| JUPC113 | $202 \pm 28^{bc}$ |
| JUPW121 | $196 \pm 18^{b}$ |
| Mock | $69 \pm 24^{a}$ |
| Water | $180 \pm 30^{b}$ |

*Values presented are mean ± standard error (n=3). Means were compared between treatments by DMRT. Different letters indicate significant differences among treatments ($p \leq 0.05$).

important chemical signalling molecule is phenolic acids, which effectively contribute towards antifungal agents and counter oxidative stress during disease infestation (Jain *et al*., 2015). Phenolics are known to be ubiquitous, and contribute more towards fungal infections in the host. The signalling molecules during defense response like salicylic acid and phenylpropanoid products are the products of these phenolic compounds. In this study, the antifungal activity was induced by the PGPR which in turn suppressed disease progression in PGPR-primed plants against *M. grisea*. Total phenolic acid content was measured, which determined the increase of such compounds in JUPW121-primed plants by ~1.5 fold, in JUPC113-primed by ~1.3 fold as compared to the mock-control. The water-control plants showed the existing phenolic acid content (Fig. 4.), indicating that the mock control plants possessed lower levels of phenolic acids compared to that of the water control. Thus early reports of defense status of phenolics were substantiated in this study. PGPR-mediated phenolic acid production and induction of disease resistance was reported by Sarma *et al*., (2002) and Singh *et al*., (2012) in chickpea against *Sclerotinia sclerotiorum* with different biocontrol agents (*Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas aeruginosa*). Also, *Pythium oligandrum*, a biocontrol fungus was able to induce defense in wheat against *Fusarium graminearum* by increased accumulation of phenolic compounds, in specific ferulic acid (Takenaka *et al*., 2003). Likewise, enzymes inducing systemic resistance were enhanced in chickpea after treatment with biocontrol agents when challenged with *S. sclerotiorum* (Jain *et al*., 2015) which is in agreement with our studies.

Chlorophyll is a pigment which is a part of the major photosynthetic machinery in plants, which produces various, assimilates for biological functions. This process in plants utilizes the light energy to generate ATP and reduced form of NADPH, utilised in further biochemical reactions (Tanaka and Makino, 2009; Tonelli *et al*., 2011; Kumudini *et al*., 2018). Therefore chlorophyll plays a major role in plants' wellbeing, growth and yield of the

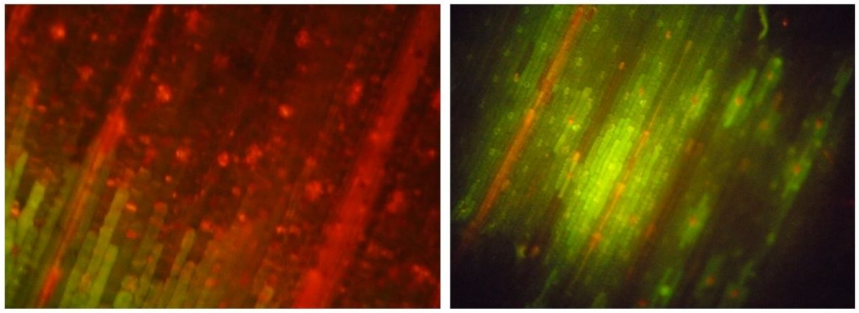

**Fig. 5 :** Fluorescence micrographs of the lesions indicating cell death by dual stains, acridine orange-ethidium bromide. Necrotic and dead cells fluoresce orange-red and living healthy cells fluoresce green at 100x magnification.

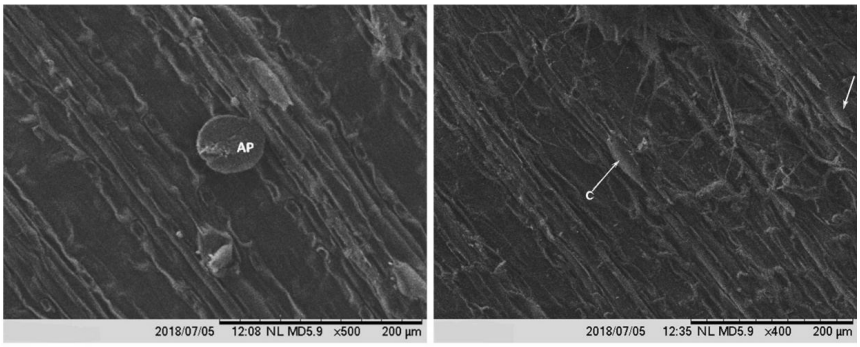


**Fig. 6 :** Scanning electron micrographs of the disease infested lesions of the leaves after 72 hpi. AP indicates appressorial structures and arrows indicate the conidial structures.

crop depending on the photosynthetic activity in turn on the amount of chlorophyll present. In our study, it was observed that chlorophyll content was not altered in the PGPR-primed plants and was also evident that pathogen-challenged control (mock-control) showed decreased levels of chlorophyll content (Table 2). This indicates that the chlorophyll content altered during disease infestation has led to decreased plant growth, thereby reducing other defense molecules in the mock-control plants.

Further to examine the pathogen-induced cell death in the primed and control plants, a dual staining technique was used. Observations under fluorescence microscope showed that all cells take up acridine orange and fluoresce green, where as necrotic cells stained with ethidium bromide appears fluoresce orange-red, due to DNA damage (Ciniglia *et al*., 2010). The leaves thus stained showed dual stain, but the cells are more orange-red in mock-control and where as green in primed leaves (Fig. 5.). Scanning electron microscopic studies showed pathogen progression on challenge inoculation in the leaf tissues of finger millet. Observations showed that the conidial and appressorial structures could be seen at a magnification of 500x and 400x respectively (Fig. 6.). The appressorium so formed penetrated the host cells through a tube-like structure called haustoria thereby infecting the adjacent cells. Thus early observations of pathogen infestation can be mitigated and thereby inhibits pathogen progression. The primed plants showed reduced symptomatic lesions on the leaves on challenge inoculation when compared to that of the un-primed mock-control plants.

Thus the present study has evidenced the induction of resistance in finger millet plants by seed priming against blast pathogen *M. grisea*, which has become the most effective approaches in induction of resistance in the present scenario (Sekar *et al*., 2018). Therefore, the PGPR which have high antifungal and plant growth promoting traits showed better performance in inducing resistance under greenhouse conditions thereby limiting pathogen progression. This was substantiated by the biochemical determination of total phenolic and chlorophyll content which portrayed the importance of seed priming. Hence seed priming by promising PGPR influence the growth and crop yield, limiting pathogen ingression, which has can be studied in detail by various omics approaches along with an understanding of the signalling cascade involved in evading the pathogen.

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